UNIVERSITY OF PENNSYLVANIA

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GERTRUDE SANDERS FRIEDMAN

A THESIS

IN PHYSIOLOGY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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AN EXAMINATION OF CEREBROSPINAL FLUID FOR OXYTOCIC ACTIVITY AS TESTED BY THE RABBIT UTERINE FISTULA PREPARATION

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With the possible exception of the Guggenheim (1914) test there is at present no chemical method of detecting the presence of secretions of the posterior lobe of the pituitary gland. Investigators have, therefore, utilized the known physiological actions of extracts of this gland in testing for its secretions in body fluids. Pressor, diuretic-antidiuretic, melanophore expanding, and oxytocic properties of cerebrospinal fluid have all been reported and assumed to be due to the secretion of the posterior pituitary directly into the fluid (Cow, 1915; Cushing and Goetsch, 1910; Jacobson, 1920; Dixon, 1923; Houssay and Ungar, 1924; Trendelenburg, 1924; Jánossy and Horváth, 1925; Miura, 1925; Trendelenburg, 1926; Mestrezat and Van Caulaert, 1926; Ehrhardt, 1927; Van Dyke and Kraft, 1927; Hoff and Wermer, 1928; McLean, 1928; and Sato, 1928, among others).

In spite of the work of Van Dyke (1926) indicating that the melanophore factor is more concentrated in the pars intermedia than in the pars posterior, it has thus far been found impossible to separate the pressor, renal, and melanophore factors. However, the presence of any or all of these in C. S. F. does not prove a similar path of secretion for the oxytocic factor since the latter has been shown to be chemically separable from them (Kamm, et al., 1928).

C. S. F. from humans and dogs has been tested on isolated strips of guinea pig or mouse uterus and reported to contain an oxytocic principle by the workers listed above. The results of those who gave definite values for the concentrations of the substance are shown in table 1. It has also been reported by some investigators (Cow, 1915; Dixon, 1923; Dixon and Marshall, 1924; Blau and Hancher, 1926; Siegert, 1927; Hoff and Wermer, 1928a; Karplus and Peczenik, 1930) that after the "hypophyseal secre-

[1] Presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Pennsylvania.

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tion" had been stimulated by various physiological or artificial procedures, the oxytocic factor was found in increased concentrations in the C. S. F. (or that it could then be detected in fluid where the method used did not indicate its presence normally). Although McLean (1928) obtained oxytocic values for C. S. F., they were lower than those he found for venous blood, and he, therefore, felt that the route of secretion is into the blood stream, the material being absorbed from there into the C. S. F.

Recently, however, Van Dyke, Bailey and Bucy (1929) have shown that increases of the $[Ca^{++}]$ of the bath surrounding an isolated uterine strip may evoke a response which exactly simulates the addition of pituitrin. They, therefore, believe that the oxytocic activity reported in C. S. F. by other workers is due solely to the fact that the $[Ca^{++}]$ of the fluid was higher than that of the bath to which it was added.

TABLE 1

Concentration of oxytocic substance in C. S. F. reported by previous workers

The values in this table are calculated on the assumption that 3 mgm. fresh gland = 1 international unit and that "Pituitrin" contains 10 international units per cubic centimeter.

NAME	REFERENCE	TYPE FLUID	INTERNATIONAL UNITS PER CUBIC CENTIMETER
Dixon	1923	Dog cisternal	0.02–0.2
Trendelenburg	1924	Cat cisternal	0.0001 average
Miura	1925	Cat cisternal and lumbar	0.00013 average
Van Dyke and Kraft	1927	Dog cisternal	0.00007–0.0007
		Human lumbar	0.0004 average
McLean	1928	Dog cisternal	0.000002–0.00024
			0.00002–0.00003 usually

The development of the rabbit uterine fistula technique (Reynolds, 1930) offered a way to escape the difficulties inherent in isolated tissue experiments. By injecting intravenously, the buffers of the rabbits' blood are utilized to prevent radical ionic changes in the uterus and the solutions do not have to be accurately buffered or neutralized.

Previous work on the preparation has shown (Weinstein, Reynolds and Friedman, 1931) that the injection of foreign proteins evokes no response in the uterus unless the rabbit has been previously sensitized to the protein used. In other experiments (Weinstein and Friedman, unpublished) no uterine reaction has been obtained when as much as 8 cc. calcium gluconate (= 80 mgm. Ca) have been injected at one time. The fistula does not respond to injections of 0.2−0.6 mgm. of histamine although 0.5−1.3 mgm. per kilo body weight is the lethal dose in the rabbit.

The fistula thus affords a uterine preparation which is relatively, at least,

insensitive to changes of ionic concentration (including Ca ions) or the addition of protein or histamine. With this rather specific test object the question of the presence of the posterior pituitary oxytocic factor in the C. S. F. of dogs and humans has been reinvestigated.

METHODS. 1. *Animals.* The rabbits used were all post partum animals as these have been shown (Reynolds, 1931) to have better uterine activity and to be more sensitive to pituitrin.

2. *Uterine fistulae* were made from 1 to 18 days post partum (usually within 5 days) with the technique and precautions described by Reynolds (1930) and Reynolds and Friedman (1930). The animals were first used for recording not less than three or four days after operation.

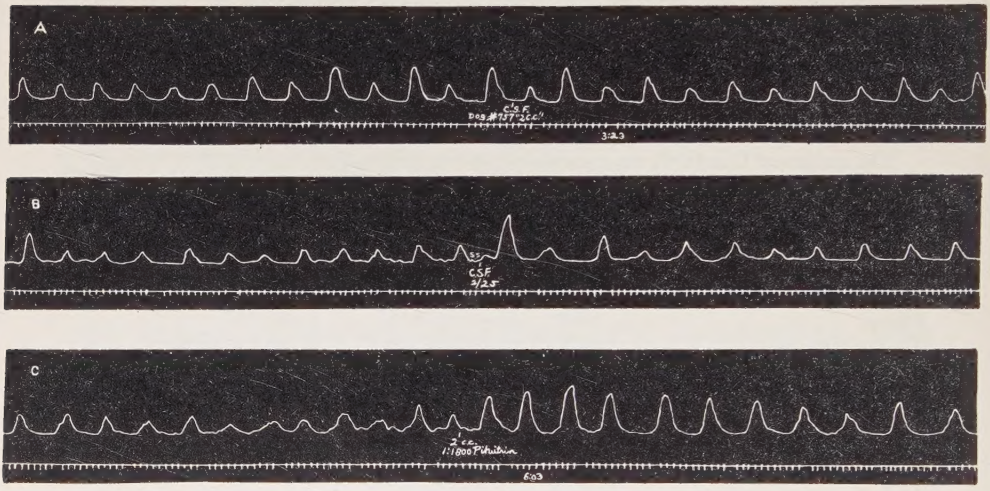

Fig. 1. A. Negative result of injection of 2 cc. dog C. S. F.

B. Negative result of injection of extract of human C. S. F. representing original volume of 92 cc.

C. Result of later injection in same experiment as A and B of 2 cc. 1:1800 pituitrin. Response shows less effect on tonus and more on size of contraction than usual response to small doses.

Time: 5 seconds. One-fourth original size.

3. *Experiments* were recorded as described by the above workers. The sensitivity of the rabbit was checked before and after the injection of fluids by the administration of known amounts of pituitrin. All injections were made intravenously at intervals of not less than ½ hour.

4. The *standard* of comparison was "Pituitrin" or "Pitocin."[3] Each of these preparations contained ten international oxytocic units per cubic centimeter. The potency of two bottles of "Pituitrin" was checked in our laboratory against a solution of standard pituitary powder from the U. S. Hygienic Laboratory and found to

[3] These extracts were very generously furnished through the courtesy of Dr. Oliver Kamm and Dr. E. P. Bugbee, by the research department of Parke, Davis & Co.

contain ten international units per cubic centimeter within the limits of accuracy of our method of assay. All dilutions were made just before using, in 0.9 per cent NaCl solution.

5. *Cerebrospinal fluids.* a. *Dog.* The fluid was collected by cisternal puncture from dogs anesthetized with ether after subcutaneous morphine and atropine. The fluid used was usually macroscopically clear (see table 2) but a control showed (see below) that bloody fluid could also be used.

b. *Human.* Human fluid was obtained[4] by lumbar puncture (local anesthesia) from both male and female psychopathic patients (maniacs, paretics, alcoholics, etc.).

TABLE 2

Experiments with dog cerebrospinal fluid

DOG NUMBER	TIME C. S. F. KEPT	DESCRIPTION OF C. S. F.	C. S. F. INJECTED (NO RESPONSE)	RESPONSE TO PITUITRIN INJECTIONS OF	THEREFORE C. S. F. MUST CONTAIN < INTERNATIONAL UNITS PER CUBIC CENTIMETER
			cc.		
1	13 days	Bloody	1.0	1.0 cc. 1:1000	0.01
1	3 days	Clear	2.0	2.2 cc. 1:500	0.022
2	3 days	Clear	2.0	2.2 cc. 1:500	0.022
3	7 days	Clear	2.9	3.2 cc. 1:800	0.014
4	13 days	Clear	2.0	2.0 cc. 1:1800	0.0055
5	6 days	Somewhat bloody	2.0	2.0 cc. 1:500	0.02
6*	6 days	Clear	2.0	2.0 cc. 1:500	0.02
7*	1 day	Clear	2.0	2.0 cc. 1:500	0.02
8*	2 days	Clear	2.0	2.0 cc. 1:500	0.02
3	2 hours	Clear	2.0	2.0 cc. 1:1000	0.01
4		Tinged			
6		Clear			
9		Tinged			
7	6 hours	Clear	2.0	2.0 cc. 1:500	0.02
10		Clear			
8		Clear			
11		Clear			

* These samples are also included in the composite fluids.

The fluid (dog or human) was collected into tubes packed in ice, was immediately acidified to approximately 0.25 per cent with acetic acid, and was kept on ice until used. Just before injection the fluid (or extract) was roughly neutralized with $NaHCO_3$ in order to prevent irritation of the rabbits' veins.

RESULTS. The enormous difference between the values reported by previous workers for the oxytocic material in C. S. F. is obvious from table 1. It has been possible to cover the highest figures (given by Dixon)

[4] For the large amounts of human fluid required we are greatly indebted to the Staff of the Psychopathic Department of the Philadelphia General Hospital without whose coöperation this part of the work would have been impossible.

using unconcentrated dog fluid. As this has, at times, taxed the sensitivity of the method, experiments have also been undertaken in which large amounts of human fluid have been concentrated and extracted before injection.

A. *Unconcentrated dog cerebrospinal fluid.* The results with unconcentrated dog fluid are summarized in table 2. It will be noted that in each case the C. S. F. had no effect although the uterus responded to doses of pituitrin equal to or less than that which should have been contained in the fluid if the minimal amount reported by Dixon were present.

In two cases the fluids injected were each composed of equal parts of samples from four dogs. The first of these (from animals 3, 4, 6 and 9) indicates that the average for the four fluids is less than *one-half* of Dixon's minimum. Since one of the four was also shown separately to be below the minimum, this is strong evidence that all were.

In the second group of four fluids (animals 7, 10, 8 and 11) the lack of response to the C. S. F. indicates that the average content of oxytocic material must be less than Dixon's minimum. Two of the samples were also shown individually to be below it. Under these circumstances it seems improbable that the other two were above it.

It may be questioned whether pituitary hormone, even if originally present, is stable in the fluids for the periods which they were kept (2 hours to 13 days). Therefore, to four different *composite* fluids, one of which was somewhat bloody, we added sufficient pituitrin to make its composition in the range of Dixon's maximal value (for greater ease in titrating). These were then compared, after varying intervals, to fresh pituitrin solutions. The difficulties in titrating these controls and the validity of the results thus obtained are fully taken up in the discussion. It may be stated here that in the control fluids kept 6, 9, 28 and 28 days any deterioration, if present, must have been less than 25 per cent. However, it must be emphasized that there was no real evidence of any loss. As one of the controls kept the longest time was macroscopically bloody, the use of somewhat bloody fluid experimentally seems justifiable.

B. *Concentrated human fluid.* Composite samples of human C. S. F. totaling 36 cc. to 92 cc. each were dried in evaporating dishes over a hot plate with an electric fan. The residue was extracted with four or five washings of 95 per cent ethyl alcohol and centrifuged to remove the salt. The decanted liquid was again dried as above and the residue extracted with 0.25 per cent acetic acid made in 0.9 per cent NaCl. Four or five washings were used, and the final volume was 1.5 to 2 cc.

A series of thirteen known pituitrin solutions made in 0.9 per cent NaCl (25 cc. amounts) was extracted by this method and the end products titrated against fresh pituitrin solutions. In eight cases the yield was 80 per cent or over; in two it was less than 65 per cent (one about 60 per cent

and one still lower); and in the remainder it was less than 75 per cent. The mode was around 80 per cent. (It is probable that the error in such titrations is at least 10 per cent in either direction. However, in view of the usual loss of sensitivity of the preparation during the experiments they were consistently conducted in such a manner that the chances for high estimations of yields were minimized while the chances for low estimations were increased.)

An at present incomplete group of three control fluids has been made by adding pituitrin to human C. S. F. (composite samples totaling 15 to 65 cc.). The original concentration in two cases approximated the maximum reported by Van Dyke and Kraft (1927), while the third had a somewhat greater pituitrin concentration. These fluids were then extracted by the method just described and the yields determined. In one case the yield was estimated as greater than 65 per cent and less than 80 per cent. A

TABLE 3

Experiments with extracts of human cerebrospinal fluid

EXTRACT NUMBER	ORIGINAL VOLUME OF C. S. F.	RESPONSE TO PITUITRIN INJECTIONS OF	THEREFORE C. S. F. MUST CONTAIN < INTERNATIONAL UNITS PER CUBIC CENTIMETER*
	cc.		
1	36	2 cc. 1:870	0.0009
2	65	1.6 cc. 1:800	0.00044
3	87	1.8 cc. 1:750	0.00038
4	90	1.9 cc. 1:750	0.00038
5	58	2 cc. 1:1800	0.00027
6	92	2 cc. 1:1800	0.00017

* Allowing for 30 per cent loss as discussed in the text.

month later on another rabbit the same extract was titrated as between 80 per cent and 85 per cent. The results from the other two extracts in this series also showed yields around 80 per cent. Since these results are in close agreement with those on the saline solutions, we feel justified in including the latter as further evidence that our method of extraction will recover in most cases 70 per cent to 80 per cent of pituitrin added to C. S. F.

In table 3 are summarized the results of the injections of extracts of C. S. F. to which no pituitrin had been added. No response was ever obtained from any fluid although in each case the whole extract was injected at one time. Yet the pituitrin responses in these experiments indicate that in five of the six cases the concentration of oxytocic substance, if present, must have been *less than* the *average* reported by Van Dyke and Kraft even if it is assumed that a loss of 30 per cent occurred during extraction. As each extract represented fluid from at least ten patients, each of these

values is comparable to the *average* values of previous workers. (Strictly speaking, they are not true averages as the amounts of fluid from different patients varied greatly.)

Discussion. The apparently nearly specific character of this method for detecting pituitrin-like material outweighs, we believe, any disadvantages in its use for problems such as that undertaken in the present investigation. Practical disadvantages are, however, present, and it is impossible to evaluate the experimental or the control data without considering the experimental difficulties encountered.

The principal disadvantages of the method lie in the low and variable sensitivity. At best the fistula is much less sensitive than the isolated strip. (In a few instances only was a response to 0.004 international unit obtained although responses to 0.008 international unit were more frequently seen.) In addition to this, enormous variations in sensitivity were seen from rabbit to rabbit. At present we can not definitely explain these differences. Moreover, it was found that the threshold for the same rabbit also fluctuated. There was usually a progressive decline in sensitivity in the days following the first use of the preparation (good fistulae were used up to 10 to 14 days after the operation).

In addition to these difficulties, there was invariably a decrease in response to pituitrin in the course of each experiment. This occurred in spite of the precaution of allowing a half hour to elapse between injections.[5] The rate of the loss of sensitivity was not a constant one even on the same day (it was liable to be much more rapid after the animal had been in use for several hours than at first). In the present experiments the sensitivity to pituitrin was, therefore, checked after each C. S. F. or control fluid injection.

In these experiments we also had two possible sources of error which are not inherent in the method but were due to the fact that we were using small, almost minimal doses of pituitrin.

The first of these is one which might easily lead to false results unless precautions are taken to avoid it. It was found that not only the amount of pituitrin injected but also the concentration influenced the response; an animal which responded to 0.4 cc. of 1:400 pituitrin did not respond to 1.6 cc. of a 1:1600 dilution although the amount of pituitrin injected was obviously the same. (This dilution effect is greater in some animals than in

[5] Other work in this laboratory (Weinstein and Friedman, to be published) has indicated that the uterine refractory period after an injection of pituitrin (reported by Frankl-Hochwart and Fröhlich, 1910; and Fuhner, 1913) lasts approximately twenty minutes after intravenous injections of the magnitude used in the present work. However, pituitrin was often injected ten minutes after an injection of C. S. F. and no change in the response was seen which could have been attributed to a tolerance due to the preceding fluid.

others and can be somewhat reduced by the use of larger hypodermic needles which allow a more rapid injection.) In the experiments reported in this paper, therefore, the volumes of the corresponding control pituitrin injections were always made equal to or greater than those of C. S. F. given. (Using these larger, more dilute injections our most sensitive preparation responded well to 0.1 international unit—1 cc. of 1:1000 pituitrin.)

The second special difficulty due to the small doses of pituitrin injected was that in some preparations spontaneous motility changes occurred which simulated the pituitrin responses obtained in these rabbits. In most of these the experiment was discarded. However, in the case of three dog fluids it was readily apparent that the change, which followed the known pituitrin injection, was a pituitrin response (and not one of these spontaneous variations) since it could be obtained repeatedly, each time with the correct latency. In addition, no such effect was ever seen following an injection of C. S. F. No C. S. F. was considered negative unless a valid pituitrin response was obtained *after* the fluid was injected.

It is due to these difficulties that it has been impossible to obtain more accurate titrations of the controls for the preservation of pituitrin in dog fluid since the loss of sensitivity happened to be particularly rapid in the animals of this series. Nevertheless these experiments do clearly show that if there was any deterioration in the control C. S. F., it was not greater than 25 per cent. For example: 0.5 cc. of a 1:10 dilution of pituitrin was added to 10 cc. of a composite sample of dog C. S. F., making a pituitrin dilution of 1:200. This control C. S. F. was then kept 28 days before being titrated. The early part of the titration showed no difference between suitable amounts of this control fluid and of fresh pituitrin solutions. It was then found that the response to 0.3 cc. of a fresh 1:200 pituitrin solution was quite comparable to the next injection which was of 0.4 cc. of the control C. S. F. (made as a 1:200 dilution originally). This might have indicated a 25 per cent deterioration in the control fluid were it not for the fact that the final injection of 0.4 cc. of a fresh 1:200 pituitrin solution now gave a response only slightly, if at all, larger than that previously given by the injection of 0.3 cc. of the same solution. This showed a loss of sensitivity in the preparation. This loss might just as well have occurred between the time of the 0.3 cc. of the fresh pituitrin solution and the control fluid as between the latter and the final fresh solution injected.

When the saline solutions of pituitrin were extracted, sufficient pituitrin could be used to make the final extract relatively concentrated and thus allow more dilutions. Consequently, it was possible to obtain more accurate titrations even though the sensitivity of the fistula had decreased during the experiment. With extracts of pituitrin in human C. S. F. the entire amount, representing an initial volume of 15 to 65 cc., usually had to be injected at once in order to evoke a response. The matching of the response by a fresh pituitrin solution was therefore more difficult and more liable to error from variations in sensitivity of the preparation. There was however no evidence that the loss of pituitrin was any greater in the extracts of C. S. F. than in those of saline solutions (20 per cent to 30 per cent).

The controls thus show that pituitrin will keep in C. S. F. under our experimental conditions whether the fluid is used as obtained or is extracted. In the first series of experiments (with unconcentrated dog C. S. F.) the uterine preparations should have given a response if the experimental fluids contained Dixon's minimal concentration of oxytocic substance (or less in some cases). We, therefore, wish to advance the data in this paper as evidence that there is no pituitrin-like material in the C. S. F. of the dogs of our laboratory in amounts approaching those postulated by Dixon. As can be seen from table 1, other workers reporting the presence of an oxytocic substance in C. S. F. have all found it in a much smaller concentration than did Dixon. Blau and Hancher (1926) in an attempt to repeat Dixon's experiments were unsuccessful at least so far as normal fluid was concerned. The data obtained by our method thus confirm these previous investigators.

Whether the pituitary normally secretes into C. S. F. or whether Van Dyke, Bailey and Bucy (1929) are correct in their belief that the oxytocic effect reported by others is due to a Ca ion concentration difference, we can not at present say. Our preliminary experiments on concentrated C. S. F. showed that the amount of pituitrin in the original fluid, if present at all, must have been less than 0.0009, 0.00044, 0.00038, 0.00038, 0.00027, and 0.00017 international unit per cubic centimeter. Since the average value reported by Van Dyke and Kraft (1927) was 0.0004 international unit per cubic centimeter (see table 1) this indicated that we could find no pituitrin-like material in C. S. F. in amounts approximating those reported by these workers. Three possible explanations for this negative result occur to us.

First, the pituitary may secrete into C. S. F. but in still smaller amounts so that the concentration of the secretion more nearly approximates those reported by Miura, Trendelenburg, and McLean. We hope to enlarge our series using still larger amounts of C. S. F. in order to cover the full range of values reported by all these workers.

Secondly, it is theoretically conceivable that the true pituitary hormone differs sufficiently in chemical properties from pituitrin to lead to its destruction by the method of extraction used in this work even though pituitrin is not destroyed. We believe that this is highly improbable.[6]

[6] It should be pointed out that the human fluids used were obtained from cases in which there was no clinical evidence that the hypophysis or its path of secretion was in any way abnormal due to nearby neural lesions. This, we believe, eliminates the basis for the objection raised by Carlson and Martin (1911) to the work of Cushing and Goetsch (1910) who used patients showing hypophyseal and neural disturbances. The further objection raised by these workers—namely, that the final fluid was a highly concentrated salt solution -we have met by the alcoholic extraction, which removes practically all of the salt present.

The third possibility is that the oxytocic activity of C. S. F. previously reported was due to something other than the posterior pituitary hormone, presumably to a Ca ion effect. We regard this as the most plausible explanation of our results. We hope to be able to show this more definitely by the further work mentioned above. Attempts will also be made to increase the oxytocic factor by the injection of the various substances used by previous workers or by the use of fluid from pregnant animals and to determine if the effect is due to increased hypophyseal secretion as they believed.

SUMMARY

1. The use of the rabbit uterine fistula as a test for the presence of pituitrin is described. The advantages and disadvantages of the method are discussed.

2. Using the acidified cisternal fluid of normal dogs it has been found impossible to show the presence of a pituitrin-like oxytocic material in the amounts reported by Dixon for such fluids.

3. A method of concentrating and extracting dilute solutions of pituitrin is described.

4. Preliminary experiments on large amounts of fluid from psychopathic patients (lumbar puncture) extracted by this method have failed to reveal any oxytocic material in amounts approximating those found by Van Dyke and Kraft.

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